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Award Number: DAMD17-00-1-0518

TITLE: Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

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REPORT DATE: August 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Final (1 Jul 2000 - 26 Jul 2004)

4. TITLE AND SUBTITLE
Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

5. FUNDING NUMBERS
DAMD17-00-1-0518

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Serine-dependent carboxylesterases (E.C.3.1.1.1) are found in a variety of tissues with high activity detected in the human liver. Carboxylesterases (CaE) hydrolyze aliphatic and aromatic esters, and aromatic amides; and play an important role in the detoxification of xenobiotic chemicals that contain organophosphate (OP) compounds. Thus, an injectable form of human hCaE should prove to be a valuable antidote for protecting soldiers from these chemical agents. The goals of this project were to over express a functional human liver hCaE from a recombinant cDNA in a human cell line, and isolate and purify the recombinant protein. To accomplish these goals, the cDNA encoding hCaE was altered in order to convert it to a secretory form. Expression of the site-mutated cDNA in cell culture resulted in the secretion of an active hCaE into the growth medium. Thus, the secreted hCaE enzyme was concentrated and purified using hydrophobic interaction chromatography, and isoelectric focusing chromatography. The long-term goal of the project is to isolate quantities sufficient for crystallization with organophosphate agents. To this end, we were able to purify and ship 130 mg of >90% electrophoretically pure enzyme to the USAMRICD to be used for further experimentation.

14. SUBJECT TERMS
Carboxylesterase; cDNA cloning; cell culture; protein secretion; enzyme purification; orgaophosphate detoxification;

15. NUMBER OF PAGES
38

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Re: Final progress report: DAMD17-00-1-0518 - Expression and Purification of a Potential Antidote for Organophosphate Warfare Agents

Kenneth D. Lanclos, Ph.D., Principal Investigator

Introduction:

The serine-dependent carboxylesterases (E.C. 3.1.1.1) are found in many different species (1-8), and in a variety of tissues (9-14), with high activities detected in the liver. Generally, carboxylesterases exist as 60 kDa monomers, but a few associate to form homotrimers of approximately 180 kDa (1-4). cDNA clones have been obtained by screening lambda gt11 expression libraries. Their screenings have resulted in the expression of five isoenzymes of rat liver carboxylesterase (5-9) and at least two isoenzymes from the human liver (10-12). The cDNA sequences are, generally, 1.7 to 1.9 kb in size, and they encode mature proteins that range from 507 to 568 amino acids. The different isoenzymes of liver carboxylesterases are all N-linked glycoproteins of the high mannose type (5,6,12,13). Core glycosylation of the carboxylesterases occurs in the endoplasmic reticulum lumen (14,15), and, thus it is necessary to stabilize the active conformation of the protein (6,16). Whereas, most glycosylated proteins are secretory, the carboxylesterases are localized to the luminal side of the endoplasmic reticulum, especially in humans (4-6). Very small amounts of liver carboxylesterase, however, are present in the serum (17). Carboxylesterases can hydrolyze a variety of substrates in the serum, including aliphatic and aromatic esters, and aromatic amides (18). An important function of these enzymes may be the hydrolysis, and subsequent detoxification, of pesticides, insecticides and drugs (18), many of which contain organophosphate compounds that bind covalently to the active site of the enzymes (19,20). In this regard, the carboxylesterases generally function as a high affinity-low capacity detoxification mechanism, in which organophosphates react in an irreversible 1:1 stoichiometry (17). Thus, the detoxifying ability of carboxylesterase is limited by its low concentration in serum where it encounters organophosphate compounds. A treatment for organophosphate toxicity is the administration of oximes, which reactivate inhibited acetylcholinesterase and restore cholinergic neurotransmission (17). Species with high levels of serum carboxylesterase, such as rats and mice, achieve a higher level of oxime-induced reactivation of organophosphate-inhibited acetylcholinesterase than species with

lower levels of carboxylesterase; this suggests that oximes also reactivate organophosphate-inhibited carboxylesterase (17). This recycling of organophosphate-inhibited carboxylesterase provides additional protection by making the enzyme available for further binding to organophosphates, and thus, increased detoxification.

The goals of this project were to over express a functional human liver hCaE from a recombinant cDNA in cell culture, isolate the enzyme, and purify the recombinant protein. To accomplish these goals, the cDNA encoding hCaE was altered in order to convert it to a secretory form. Expression of the site-mutated cDNA in cell culture resulted in the secretion of an active hCaE into the growth medium. Thus, the secreted hCaE enzyme was concentrated and purified using hydrophobic interaction chromatography, and finally purified using preparative isoelectric focusing chromatography.

Body:

1. Construction of expression vectors:

A full length cDNA clone of human liver carboxylesterase (GeneBank accession # 55509- kindly provided by D.L. Long and F.J. Gonzales, NIH))was subcloned into the baculovirus tranfer plasmid, pVL 1393 (Invitrogen, San Diego, CA). [This work was completed at the Institute for Chemical Defense, Aberdeen Proving Ground, Maryland]. The recombinant plasmid, pRc/CMV-hCaE, was prepared by substituting the 2 kb EcoRI fragment from pVl1393 for the 0.9 kb EcoRI fragment in pc/CMV. Two additional mammalian expression vectors were prepared. The recombinant plasmid, pRc/CMV-hCaE, was prepared by substituting the 2kb EcoRI fragment from pVL 1393 for the 0.9 kb EcoRI fragment in pRc/CMV. A mammalian expression vector, pBXG1 (Ptashne, M), containing the Simian Virus 40 enhancer/promoter (SV40) and splice/ polyA signals was a gift from Dr. T. Towns, Birmingham, Ala. The pSV40 mammalian expression vector was prepared as a modification of the plasmid pBXG1 by removing the GAL4 DNA binding domain with *Hind*III/*Xba*I and inserting a multiple cloning sequence. The *Eco*RI cDNA fragment from pVL1393-hCaE, was subcloned into a unique *Eco*RI site in pSV40 to produce the recombinant expression plasmid, pSV40-hCAE.

2. Expression of recombinant hCaE:

The expression vectors described above were used to afford a comparison of the expression of hCaE in Sf9 insect cells, simian COS- 7 cells, and human kidney 293T cells. Following infection and/or transfection we have shown significant expression and processing of an enzymically active carboxylesterase in insect Sf9 cells as well as in mammalian cell types. The recombinant enzyme was found to be effective in the hydrolysis of alpha-napthyl acetate and p-nitrophenylbutyrate. In addition, the activity of the recombinant carboxylesterase was not inhibited by the general esterase inhibitor physostigmine. The SV40-hCaE vector containing the powerful simian virus promoter was used for transfection of the simian cell type, while the cytomegaloviral promoter was chosen for transfection of the human cell lines. Similar levels of carboxylesterase activity were observed in both the insect cells and mammalian cells (fig.1).

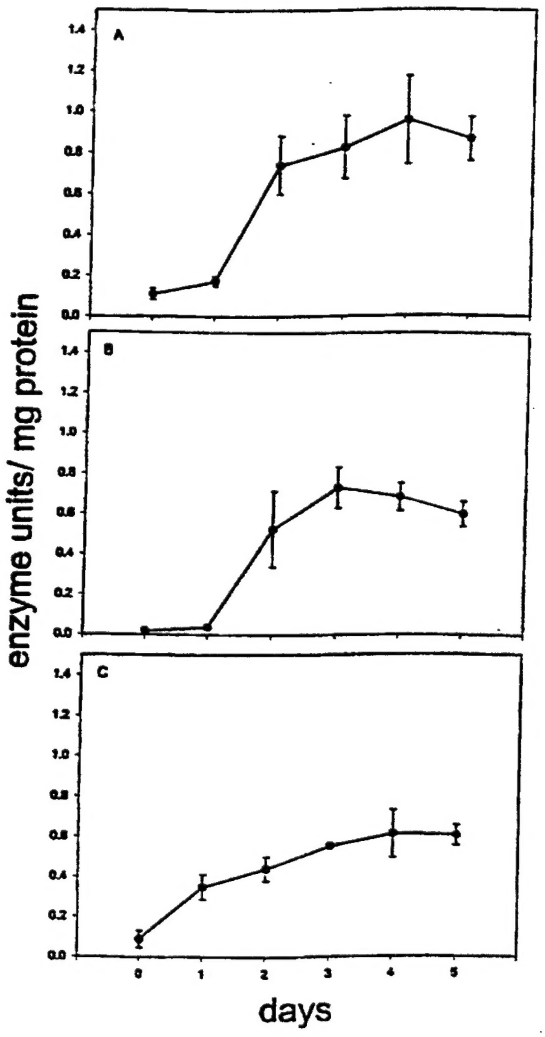

Figure 1: Kinetics of hCaE production in different cell types

Following the introduction of the appropriate expression vector, cell extracts were prepared at the indicated times and assayed for carboxylesterase activity. Each point represents the mean +/- std err. From three separate infections or transfections. (A) Sf9 insect cells infected with baculovirus bearing hCaE cDNA. (B) COS7 cells transfected with plasmid pSV40-hCaE. (C) 293T cells transfected with the plasmid pRc/CMV-hCaE.

However, the expression in mammalian cells was significantly higher since the transfection efficiencies for the mammalian cells was in the range of 20-30% as compared to over 90% in the baculoviral infections. In all cases, the level of enzyme reached a steady state in culture between two and three days of incubation with >95% of the enzyme remaining as intracellular enzyme. Thus it appears that the half-life of the

enzyme is 24 to 36 hours in each of the cell lines studied. As might be expected, the level of recombinant enzyme available for isolation and purification is limited to the number of successfully transfected cells and/or the nutrients that are available for continued growth of these cells.

The pattern of expression of the hCaE cDNA in each cell type was examined by zymograms produced by isoelectric focusing followed by staining for carboxylesterase activity. Multiple bands of the enzyme were observed in the mid range pI. (fig.2).

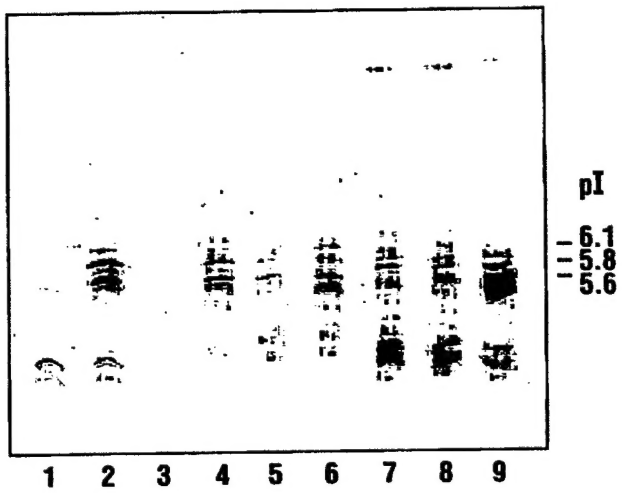

Figure 2: Isoelectric focusing patterns of cell extracts

Extracts were prepared from cultured cells four days after the introduction of the appropriate vector with (lanes 2, 4, 6 and 8) or without (lanes 1, 3, 5 and 7) the hCaE cDNA insert. Samples were focused on an IEF gel, pH 305-9.5 and stained for esterase activity. Lanes 1 and 2, Sf9 cells; Lanes 3 and 4 COS7 cells; Lanes 5 and 6, 293T cells; Lanes 7 and 8, HepG2 cells, and Lane 9, human liver extract.

A major goal of these studies was to produce a recombinant carboxylesterase that has the potential for use as an antidote against the action of soman (methyl pinacolylphosphonofluoridate) and other toxic organophosphorus agents. For this purpose it was important that the recombinant enzyme, unlike the cholinesterases, possess a relatively specific esterase activity and that the recombinant enzyme be regenerable by oxime agents. In this regard, carboxylesterase can be differentiated from cholinesterase [20] by the inability of carbamates to inhibit carboxylesterase at concentrations that readily inhibit cholinesterase [19]. In addition, carboxylesterase can be differentiated by its ability to be reactivated by oximes after inhibition by organophosphorus compounds; cholinesterase cannot be reactivated after inhibition by organophosphorus compounds. Physostigmine is the carbamate most commonly used to differentiate carboxylesterase from cholinesterase [17] and soman is the organophosphorus compound that has the greatest difference in oxime reactivation to

differentiate inhibition of carboxylesterase and cholinesterase [19]. Thus, our findings show that the recombinant cDNA of human liver carboxylesterase codes for an enzyme that is processed in the Golgi to produce an active form of the enzyme in insect, simian, and human cells. Most importantly, the recombinant carboxylesterase is not inhibited by physostigmine and is almost completely regenerable by 1mM diacetylmonoxime, an oxime (see Table I).

Table I: Inhibition and reactivation of hCaE in extracts from different cell cultures

	Carboxylase Activity (% of Control)[a]			
Treatment	**Sf9**	**COS7**	**293T**	**HepG2**
Physostigmine[b]	95+/-4	97+/-3	96+/-2	98+/-3
Soman[c]	5+/-3	7+/-4	8+/-4	4+/-3
Oxime[d]	93+/-5	89+/-7	91+/-4	94+/-5

Extracts from the different cell types were each treated with physostigmine, soman and oxime, to determine if the recombinant enzyme functions in the same way as native enzyme isolated from human liver.

[a] Activities (means ± std. err.) are expressed relative to untreated extracts.

[b] Incubation with 10μM physostigmine for 30 minutes at 25°C (pH 7.4).

[c] Incubation with 1μM soman for 30 minutes at 25°C (pH 7.4).

[d] Incubation of soman-inhibited carboxylesterase with 1mM diacetylmonoxime for two hours at 25°C (pH 7.4)

3. Cloning and expression of a secretory form of human liver carboxylesterase:

The expression experiments described above had shown that our recombinant (pRC/CMV-hCaE) plasmid construct, when stably integrated into 293T cells, successfully expressed a recombinant and active form human liver carboxylesterase (hCaE) in culture. Unfortunately, however, the enzyme was found to be mostly membrane bound and showed levels of expression that were directly proportional to the number of cells in culture. Therefore, in order to obtain meaningful levels of enzyme, it was deemed necessary to over express a functional human liver CaE from the recombinant cDNA, as well as to successfully isolate and purify the active recombinant enzyme. To accomplish these goals, the C-terminal retrieval signal of human liver hCaE, HIEL (his-ile-glu-leu), was mutated in order to convert the native membrane bound protein into a secretory form of carboxylesterase. A new recombinant plasmid, pRc/CMV-mhCaE, was constructed (fig.3) and stably integrated into the

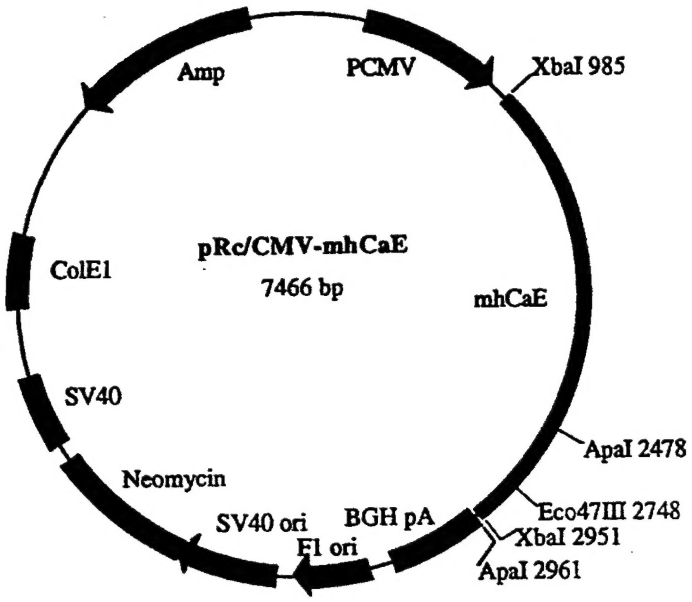

Figure 3: Expression vector containing the secretory carboxylesterase

The cDNA fragment was inserted into the expression vector at a single XbaI site located at position 985. The presence of the carboxy-terminal mutation is shown at position 2748.

genome of human 293T cells. Expression of the mutated human hCaE gene in 293T cells resulted in synthesis, processing through the golgi, and secretion of an active form of hCaE into the culture medium (fig. 4). For details of the construction of the secretory form of human liver carboxylesterase, expression in cell culture, and purification procedures see ("Expression and Partial Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase"(21) - Appendix).

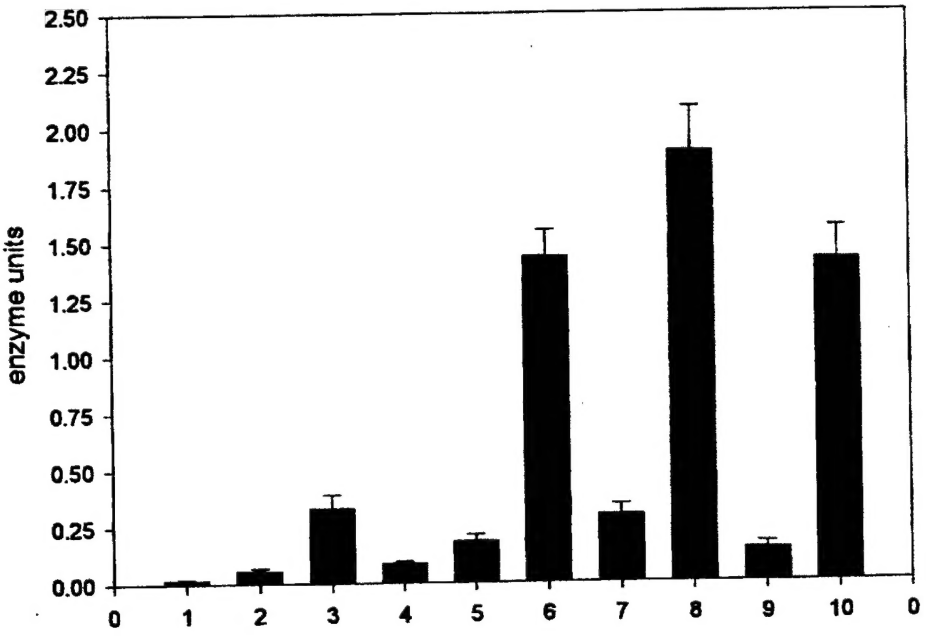

Figure 4: Stable expression of carboxylesterase
Intracellular enzyme levels (green bars) were compared to extracellular enzyme levels (red bars) for each of three mutant clones (clone A, lanes 5 and 6; clone B, lanes 7 and 8; clone C, lanes 9 and 10), as wll as for 293T cells stably expressing normal human liver carboxylesterase cDNA (lanes 3 and 4), and 293T cells that were not transfected (lanes 1 and 2). Each bar shows the mean +/- s.e. Mean of nine experiments.

At least ten enzyme units of hCaE were obtained from each ten milliliters of growth medium, and the secretory form of the enzyme displayed isoelectric focusing patterns similar to those of the native enzyme with no observable changes in activity.

4. Purification Protocols:

Stable clones of 293T cells, stably transfected with the plasmid pRC/mhCaE were placed in liquid culture for expression of the secretory form of human liver carboxylesterase. Carboxylesterase activity was observed to increase as early as 24 hours and to reach a maximum at day four (21). Cultured cells were separated from the media by low speed centrifugation and the pooled media from several cultures was used for the isolation of purification enzymatically active enzyme.

Carboxyleasterase was precipitated from the pooled media and precipitated using 70% saturated ammonium sulfate. The precipitate from the ammonium sulfate fraction was dissolved in 20 mM Hepes, pH 7.0. The crude enzyme material was then subjected

to a variety of protein isolation procedures to obtain relatively pure enzyme. The procedures that were tried included Gel Filtration Chromatography, Lectin Affinity Binding, High Performance Liquid Chromatography, Ion Exchange Chromatography, Dye-Ligand Affinity Chromatography, and Immunoaffinity chromatography. Unfortunately, all of the procedures resulted in considerable loss (60-80%) of enzyme due to binding of the enzyme to the various matricies.

Since carboxylesterase binds to many matrices used in standard protein purification procedures, we developed a liquid isoelectric focusing preparative system.(For details see "Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase"(22)- Appendix).

5. Large Scale Production of Enzyme:

During the last year, we have altered our culture conditions in order to increase the production of purified recombinant carboxylesterase. To this end, a chemostat culture system was implemented. This consisted of a culture vessel with a sterile vent and ports for spent media removal and for the addition of fresh media. 100 ml of media was placed into the culture vessel and log phase 293T cells were added. A constant volume of 100 ml of media was maintained in the culture vessel. Fresh media was pumped into the culture vessel and spent media was pumped out into a refrigerated sterile flask at a flow rate that yielded about 120 ml of spent media per day. Approximately four and one-half liters of collected media was used for further isolation of carboxylesterase. Figure 5 shows a typical isoelectric focusing chromatogram of enzyme isolated by this method.

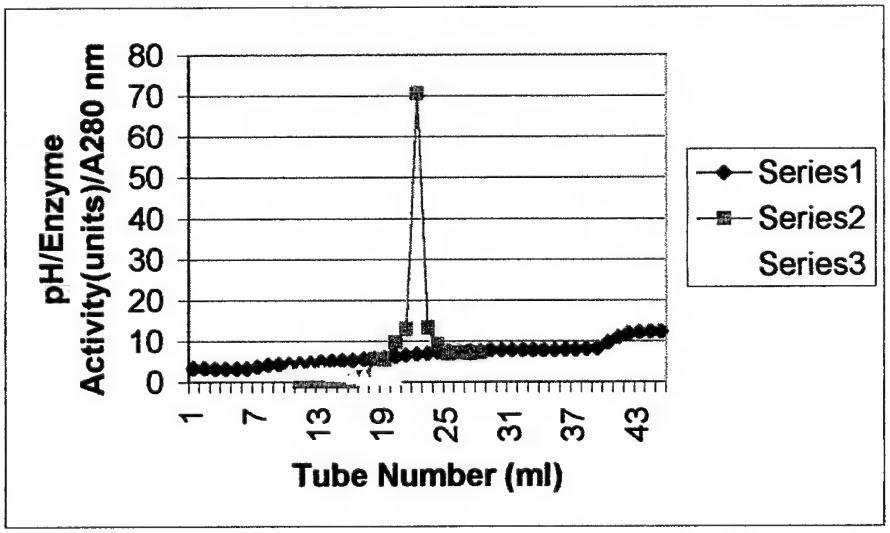

Figure 5: Preparative Electrofocusing Chromatogram

Figure 6 shows zymograms of carboxylesterase that was isolated from liquid isoelectric columns, dialyzed against 20mm Hepes, pH 7.5 and finally lyophilized for shipment to the USAMRICD to be used for further experimentation.

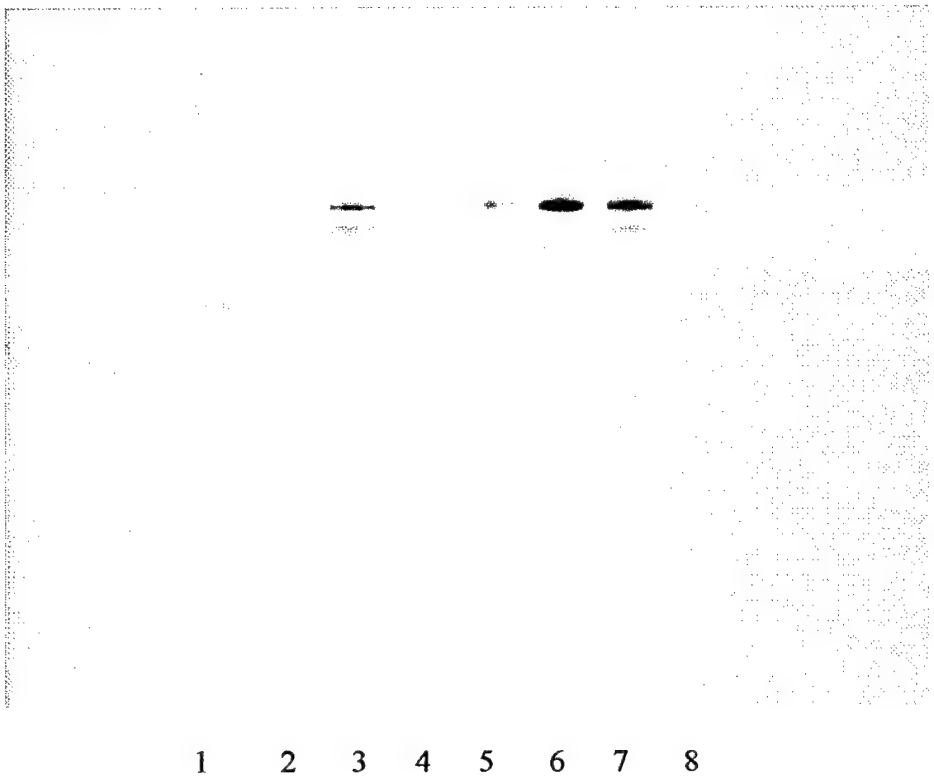

Figure 6: Nondenaturing Page Gel electrophoresis carboxylesterase isolated by liquid isoelectric focusing chromatography. Following electrophoresis, the gel was stained for ezyme activity as previously described and for protein with Coomassie blue. Lane 1; albumin standard, Lane 2; urease standard; Lane 3-8; peak fractions with carboxylesterase activity from five different representative isoelectric focusing runs.

Using this methodology we were able to send 117 mg of >90% pure recombinant human liver carboxylesterase to the USAMRICD for further experiments.

Key Research Accomplishments:

- Cloning of hCaE into bacterial, viral, and mammalian expression vectors
- Stable integration of hCaE cDNA clones into simian and mammalian cell lines for expression of active enzyme
- Conversion of hCaE cDNA to a secretory form of the enzyme by mutation of the terminal leucine (CTG) of the retention signal to that of arginine (CGG)
- Development of cell culture expression systems for secretory hCaE
- Development of biochemical methods for isolation and purification of the active form hCaE following secretion of the enzyme into culture media

Reportable Outcomes:

Manuscripts:

Maxwell, Donald M., Lanclos, Kenneth D., and Benschop, Hendrik, Carboxylesterase: Regulatory Control and Peptide-Induced Secretion of an Endogenous Scavenger for Organophosphorus Agents, Proceedings of 20th Army Science Conference vol. II, 651-655, 1997

Amanda D. Miller, David F. Scott, Terry L. Chacko, Donald M. Maxwell, John J. Schlager and Kenneth D. Lanclos. "Expression and Partial Purification of a Recombinant Secretory Form of human liver carboxylesterase" J. Protein Expression and Purification, 17:16-25, 1999

Kenneth D. Lanclos, Angela D. Soltau , Thomas D. Soltau, and Donald M. Maxwell, "Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase". In, Proceedings of the 2002 Medical Defense Bioscience Review, Hunt Valley, Maryland. U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland. 2002)

Abstracts:

Maxwell, D.M., Lanclos, K.D. and Benschop, H.P. Carboxylesterase: Regulatory Control and Peptide-Induced Secretion of an Endogenous Scavenger for Organophosphorus Agents. 20th Army Science Conference., Norfolk Virginia, June24-27, 1996.

Amanda Miller, David Scott, Terry Chacko, Donald Maxwell, John Schlager, and Kenneth Lanclos, "Expression of a Secretory Human Liver Carboxylesterase" Submitted ASBMB, Washington, May 19-20, 1998

K.Lanclos, A.Soltau, T.Soltau, D.Maxwell, "Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase", Bioscience 2002 Medical Defense Review, Hunt Valley, Maryland, June 3-7, 2002

Ph.D. Degrees:

The Doctor of Philosophy degree was supported by this award and granted to Amanda Miller at the Medical College of Georgia. The title of her dissertation is: "Expression and Purification of a Secretory Human Liver Carboxylesterase".

Personnel receiving pay:

Kenneth D. Lanclos, Ph.D. – P.I.
Angie Soltau – Research Technician
Steve Arthur – Research Technician

Cell Lines:

The following cell lines were developed during the course of this award:

- Monkey kidney cells (COS-7) stably integrated with the plasmid pSV40-hCaE,
- Human embryonic kidney (293T) cells stably integrated with the plasmid pRc/CMV-hCaE,
- 293T cells stably integrated with the plasmid pCMV-mhCaE,
- Human erythroleukemia cells (HEL) and (K562) stably integrated with pRc/CMV-hCaE,
- Sf9 insect cells infected with baculovirus bearing hCaE cDNA.

Conclusion:

Clones containing the secretory form of human liver carboxylesterase were used to establish culture conditions where the enzyme is produced maximally in the 293T human cell line. This objective was accomplished by growing cells in a chemostat where the cells are maintained in a constant exponential phase of growth. In addition, conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. Enzyme was isolated from the culture media, and the initial steps of enzyme purification using ammonium sulfate precipitation, and HIC chromatography In the final steps of purification, the use of preparative liquid isoelectric focusing chromatography was found to be productive and resulted in a preparation that was free of >90% bovine serum albumin and other minor contaminants as measure by polyacrylamide gel electrophoresis. These protocols allowed us to supply over 130 mg of >90% pure carboyxlesterase to the USAMRICD to continue crystallization studies and studies involving the detoxification of OP compounds with recombinant human liver carboxylesterase.

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Appendices:

Miller, A.D., Scott, D.F., Chacko, T.L., Maxwell, D.M., Schlager, J.J., and Lanclos, K.D. (1999), Expression and Partial Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase. *Protein Expression and Purification* **17**, 16-25

Kenneth D. Lanclos, Angela D. Soltau , Thomas D. Soltau, and Donald M. Maxwell, "Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase".(2002), In, Proceedings of the 2002 Medical Defense Bioscience Review, Hunt Valley, Maryland. U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland.

Protein Expression and Purification **17,** 16–25 (1999)
Article ID prep.1999.1121, available online at http://www.idealibrary.com on IDEAL®

Expression and Partial Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase

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Received October 5, 1998, and in revised form May 26, 1999

Serine-dependent carboxylesterases (EC 3.1.1.1) are found in a variety of tissues with high activity detected in the liver. Carboxylesterases (CaE) hydrolyze aliphatic and aromatic esters, and aromatic amides, and play an important role in the detoxification of xenobiotic chemicals that contain organophosphate (OP) compounds. The detoxifying ability of CaE is limited by its low concentration in serum where it encounters OP compounds. Studies in our laboratory have shown that a pRC/CMV-hCaE plasmid construct, stably integrated into 293T cells, expresses a human liver CaE in culture. However, the enzyme remained inside the cell and reached a low steady-state level of expression. The goals of this study were to overexpress a functional human liver CaE from a recombinant cDNA in a human cell line and to isolate and purify the recombinant protein. To accomplish these goals, a single amino acid change was made in the C-terminal retrieval signal, HIEL (His-Ile-Glu-Leu), of human liver CaE. The mutation produced a unique *Eco*47III restriction site, which aided in clone selection. The recombinant plasmid, pRc/CMV-mhCaE, was isolated and stably integrated into human 293T cells. Expression of the altered cDNA resulted in secretion of an active CaE up to levels of 500 enzyme units per liter of growth medium. Secretory CaE displayed isoelectric focusing patterns similar to those of the native enzyme with no observable changes in activity. The secreted enzyme was partially purified by hydrophobic interaction chromatography and Cibacron blue affinity chromatography. Partial enzyme purification was achieved, and CaE retained a high level of enzymatic activity. © 1999 Academic Press

Key Words: **carboxylesterase; site-directed mutagenesis; cDNA cloning; cell culture; protein purification.**

The serine-dependent carboxylesterases (EC 3.1.1.1) are found in many different species (1–8), and in a variety of tissues (9–14), with high activities detected in the liver. Generally, carboxylesterases exist as 60-kDa monomers, but a few associate to form homotrimers of approximately 180 kDa (1,2,15,16). cDNA clones have been obtained by screening λgt11 expression libraries. Their screenings have resulted in the expression of five isoenzymes of rat liver carboxylesterase (17–21) and at least two isoenzymes from the human liver (5,22,23). The cDNA sequences are, generally, 1.7 to 1.9 kb in size and encode mature proteins that range from 507 to 568 amino acids.

The different isoenzymes of liver carboxylesterases are all N-linked glycoproteins of the high mannose type (17,18,23,24) and range in molecular weight between 140 and 189 kDa. Core glycosylation of the carboxylesterases occurs in the endoplasmic reticulum lumen (25,26) and is necessary to stabilize the active conformation of the protein (18,27). Whereas most glycosylated proteins are secretory, the carboxylesterases are localized to the luminal side of the endoplasmic reticulum; especially for human (16–18). However, very small amounts of liver carboxylesterase are present in the serum (28).

Carboxylesterases hydrolyze a variety of substrates in the serum, including aliphatic and aromatic esters and aromatic amides (12). An important function of these enzymes may be the hydrolysis, and subsequent detoxification, of pesticides, insecticides, and drugs (12), many of which contain organophosphate compounds that bind covalently to the active site of the enzymes (8,29). In this regard, the carboxylesterases generally function as a high-affinity low-capacity detoxification mechanism, in which organophosphates react in an irreversible 1:1 stoichiometry (28). Thus, the

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detoxifying ability of carboxylesterase is limited by its low concentration in serum.

A treatment for organophosphate toxicity is the administration of oximes, which reactivate inhibited acetylcholinesterase and restore cholinergic neurotransmission (28). Species with high levels of serum carboxylesterase, such as the rat and mouse, achieve a higher level of oxime-induced reactivation of organophosphate-inhibited acetylcholinesterase than species with lower levels of carboxylesterase, suggesting that oximes also reactivate organophosphate-inhibited carboxylesterase (28). This recycling of organophosphate-inhibited carboxylesterase provides additional protection by making the enzyme available for further binding to organophosphates and, thus, increased detoxification. Recent studies in our laboratory have shown that a recombinant human liver carboxylesterase expressed in 293T cells, and inhibited by soman (methyl pinacolylphosphonofluoridate), could be reactivated with 1 mM diacetylmonoxime (manuscript in preparation). Thus, carboxylesterase present in serum may offer protection against toxic doses of organophosphate compounds.

Ongoing studies in our laboratory have shown that a pRC/CMV-hCaE plasmid construct stably integrated into 293T cells expresses a human liver carboxylesterase in culture. However, it was observed in these experiments that the enzyme levels reached a plateau of expression and that the enzyme was mainly intracellular and remained associated with the endoplasmic reticulum. The goals of this study were to overexpress a functional human liver carboxylesterase in cell culture and to isolate and purify the recombinant protein. To accomplish these goals, we mutated the codon for the terminal leucine (CTG) of the human liver carboxylesterase retention signal, HIEL (His-Ile-Glu-Leu) to that of arginine (CGG), as previously done on a rat liver carboxylesterase (30). The terminal G of the arginine codon was also mutated to C, to produce a unique *Eco*47III restriction site. The recombinant plasmid, pRC/CMV-mhCaE, was stably integrated into human 293T cells for expression. Results of these experiments show overexpression and secretion of an enzymatically active human liver carboxylesterase into the culture media. The secreted enzyme was partially purified and retained a high level of enzymatic activity.

MATERIALS AND METHODS

Site-Directed Mutagenesis of hCaE

Carboxylesterase cDNA (GenBank Accession No. M55509) was subcloned from a pUC vector (5) into the pNotA plasmid (5′–3′, Inc., Boulder, CO). The mutagenesis procedure was based on the work of Deng *et al.* (31) and Haught *et al.* (32) and was performed with the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene, La Jolla, CA), as described by the manufacturer.

Two primers were used for the site-directed mutagenesis reaction: a mutagenic primer and a selection primer. The mutagenic primer, 5′-TCTTCAT-TCA*GC*GCTCTATGTG-3′, produces a specific mutation at the carboxy-terminus of carboxylesterase; the codon CTG for leucine is converted to CGC for arginine. The nucleotide changes simultaneously create a novel *Eco*47III restriction site. The selection primer, 5′-CT-TGGTTGAG*GC*CTCACCAGTC-3′, mutates a unique *Sca*I site, located at position 5100 in the plasmid, to a unique *Stu*I site. The suitability of each primer was analyzed using the computer program Oligo 4.0 (National Biosciences, Inc., Plymouth, MN), and both were synthesized using a 392ABI DNA synthesizer (Applied Biosystems, Foster City, CA). Primers were purified using Sephadex G-50 chromatography (Sigma Chemical Company, St. Louis, MO), and each primer was phosphorylated as described by Sambrook *et al.* (33).

Mutant Selection

Plasmid DNA was digested with 20 units of *Sca*I (Promega) to linearize the unmutated plasmids, and the reaction mixture was used to transform XL*mut*S competent cells (Stratagene). Plasmid DNA was isolated from the transformed bacteria using the Wizard Miniprep DNA isolation procedure (Promega) and was digested with 20 units of *Sca*I. The digest was used to transform Epicurian Coli XL1-Blue competent cells (Stratagene), which were plated on LB agar plates containing ampicillin and incubated overnight at 37°C. The DNA was isolated from individual colonies as previously described and digested with 20 units of *Stu*I to confirm the presence of the mutation in the selection site. Plasmid DNA was also digested with *Eco*47III to confirm the presence of the mutation in the carboxylesterase gene. The digested samples were analyzed by electrophoresis on a 1% agarose gel.

Sequencing

The presence of the leucine to arginine mutation was confirmed by DNA sequencing (Sequenase Version 2.0 sequencing kit; United States Biochemical Corp., Cleveland, OH). The primer used for the sequencing reaction was 5′-CCCAATGGGGAAGGGCTGC-3′. a primer specific for a site 175 bp upstream of the mutated site.

Construction of the Expression Vector

DNA, isolated from 200 ml overnight cultures of bacteria (Qiagen Plasmid Maxi kit; Qiagen, Inc., Chatsworth, CA), was digested with 20 units of *Xba*I (Promega). The 1.9-kb *Xba*I fragment, containing the

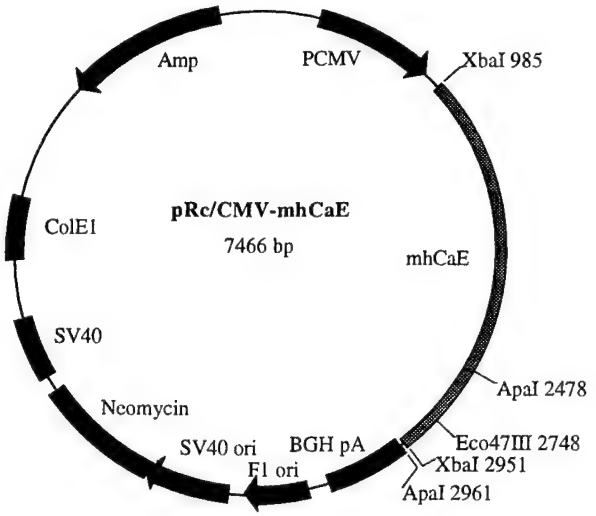

FIG. 1. Expression vector containing the mutated carboxylesterase cDNA. The cDNA fragment was inserted into the expression vector at a single *Xba*I site located at position 985. The presence of the carboxy-terminal mutation is shown at position 2748. The cDNA insert is in the forward direction relative to the CMV promoter.

full-length human carboxylesterase cDNA, was isolated using GenElute agarose spin columns (Supelco, Inc., Bellefonte, PA).

The *Xba*I fragment was ligated into the eucaryotic expression plasmid, pRc/CMV, in a 4:1 ratio using 0.1 Weiss units of T4 DNA ligase (Promega). The ligation reaction was incubated for 4 h at 16°C, and 2 μl of the 10 μl ligation reaction was used to transform competent JM109 cells (5′–3′) (33). Colonies were picked, cultures were grown overnight, and plasmid DNA was isolated as previously described. The presence of the 1.9-kb *Xba*I fragment was determined by digestion with 20 units of *Xba*I. Plasmid DNA was digested with 10 units of *Eco*47III to verify the presence of the mutation. The orientation of the cDNA relative to the CMV promoter was determined by digestion with 20 units of *Apa*I (Promega). The presence of a 1.9-kb band shows the correct orientation of the cDNA (Fig. 1).

Transient Expression of hCaE

Human embryonic kidney cells (293T) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, 100 IU penicillin–streptomycin/ml and 10 mM Hepes buffer, pH 7.5, at 37°C in the presence of 5% CO_2.

The transfection protocol was based on the work of Chen and Okayama (34). Solutions used in transfection were 2× BBS [50 mM BES (Sigma), 280 mM NaCl (Fisher Scientific, Pittsburgh, PA), and 1.5 mM Na_2HPO_4 (Fisher), pH 6.96] and 0.25 M $CaCl_2$ (33). Both solutions were sterilized by filtration using a 0.22-μm nitrocellulose filter (Nalgene, Rochester, NY).

Four different plasmids were transfected into 293T cells. The first plasmid, pRc/CMV (Invitrogen, San Diego, CA), was the expression vector without any added DNA. The second plasmid, pRc/CMV-hCaE, contained a normal carboxylesterase gene. The third plasmid, pRc/CMV-mhCaE, contained the mutant copy of the carboxylesterase cDNA. Finally, the fourth plasmid, pCMVβ (Clontech, Palo Alto, CA) (a reporter vector containing β-galactosidase), was cotransfected with each of the other plasmids in order to quantify the transfection efficiency by X-gal (Promega) staining. Mock transfections (buffer only) were done as additional controls.

On day 3 following transfection, the cells and media were collected and prepared for carboxylesterase assay. Cells were detached from the 100-mm dish; a sample was used to determine the cell viability with trypan blue and the transfection efficiency by X-gal staining (35). The cell pellet was resuspended in 1 ml of phosphate-buffered saline (PBS), pH 7, transferred to a microfuge tube, and centrifuged for 1 min at 4°C. The PBS was discarded and the cells were suspended in 500 μl of 50 mM Hepes, pH 7.5, containing 1% Triton X-100 and homogenized by grinding with a pellet pestle (Kontes, Vineland, NJ). The disrupted cell suspension, to be used for enzyme assay, was centrifuged for 1 min at 4°C and the resulting supernatant transferred to a microfuge tube.

Protein in the culture medium was precipitated by adding saturated ammonium sulfate solution to 75% saturation. The solution was allowed to stand at room temperature for 30 min and was then centrifuged at 31,000*g* for 15 min. The supernatant was discarded and the pellet was resuspended in 500 μl of 50 mM Hepes, pH 7.5.

Protein concentration in both the medium and the cell supernatant fractions was determined by using bicinchoninic acid (BCA; Pierce, Rockford, IL) as described by the manufacturer. The cell supernatant and the culture medium were assayed for carboxylesterase activity by a modification of the method of Sterri *et al.* (36). The assay buffer contained 1 mM *p*-nitrophenyl butyrate, 50 mM Hepes, pH 7.5, and 1 ml of DMSO in a total volume of 10 ml. In a cuvette, 990 μl of assay buffer and 10 μl of sample were mixed and immediately read spectrophotometrically at 400 nm, over a 60-s time interval. All reactions were linear during the 60-s reading, and data are expressed in enzyme units, μmol/min/mg protein.

Stable Expression of hCaE

Forty-eight hours after transfection, DMEM containing 400 μg/ml G418 (Gibco BRL, Life Technologies,

Inc., Grand Island, NY) was added to 293T cells transfected with pRc/CMV-mCaE. Two weeks after selection began, individual colonies were picked and each was seeded into 1 well of a 24-well plate containing 500 μl of medium. Once the individual clones reached confluence, they were seeded into a 35-mm dish and then into a 100-mm dish. The stable clones were collected and assayed for carboxylesterase expression as described above (36). For enzyme isolation and purification, stably transfected cells were grown in 175-cm^2 flasks containing 30 ml of culture medium. The cells were incubated for 72 h at 37°C in an atmosphere of 5% CO_2.

Isoelectric Focusing

Isoelectric focusing was performed using a 2117 Multiphor electrophoresis unit (LKB, Bromma, Sweden). Approximately 35 μg of protein from the cell supernatant and 200 μg of protein from media samples were loaded onto a prepackaged polyacrylamide gel containing Ampholine carrier ampholytes, pH 3.5–9.5 (Pharmacia Biotech, Uppsala, Sweden). The gel was developed according to the manufacturer's instructions (Pharmacia). After focusing, the gel was cut in half; one half was stained for protein with Coomassie blue and the other for enzyme activity with α-naphthylacetate (Sigma) as substrate coupled to Fast Red TR salt (37).

Purification Protocols

Hydrophobic interaction chromatography. The ammonium sulfate fraction from 30 ml of the culture medium was applied to an octyl Sepharose column (Pharmacia) of dimensions 5 by 1 cm. The column was equilibrated with 1 M $(NH_4)_2SO_4$, 20 mM Hepes, pH 7. Carboxylesterase was eluted from the column with 50 mM octylglucopyranoside, 20 mM Hepes, pH 8, 0.5 mM DTT. One-milliliter fractions were collected and assayed for protein and enzyme activity as described above. Fractions containing CaE activity (2–3 ml) were pooled.

Dye-ligand affinity chromatography. The hydrophobic interaction chromatography fraction was loaded directly onto a Cibacron blue Affi-Gel (Bio-Rad, Hercules, CA) column (0.8 × 15 cm) with a total bed volume of 10 ml of gel. The column was equilibrated with the hydrophobic interaction chromatography (HIC) elution buffer. Following sample application, the column was eluted with 20 ml of buffer, and 1-ml fractions were collected and assayed for enzyme and total protein. Albumin is retained on the column while CaE is eluted in the void volume.

Nondenaturing PAGE. Discontinuous polyacrylamide gels were prepared according to the manufacturer's instructions (Bio-Rad), which were based on the discontinuous buffer system of Laemmli (38). Carboxylesterase samples at various stages of purification were applied to a 4% stacking gel and separated on a 12% separating gel. In order to separate the proteins under native conditions and keep carboxylesterase in an active state, SDS was left out of the gels and running buffer. The gels were developed at 200 V, constant voltage, for 30 min. The gels were soaked in 0.1 M sodium phosphate buffer, pH 7, for 1 h and were stained for enzyme activity as described (36). The gels were rinsed with deionized water and stained for protein with a 0.1% solution of Coomassie Blue R-250. The gels were destained with a solution of 40% methanol/10% acetic acid.

Western blot. Carboxylesterase samples at various stages of purification were applied to a 4% stacking gel and separated on a 7.5% separating gel at 200 V, constant voltage, for 30 min. Following separation, the proteins were blotted onto a 0.45-μm nitrocellulose membrane (Amersham, Buckinghamshire, England) using the Mini Trans-Blot cell (Bio-Rad). Blotting was performed at 50 V for 2 h in Towbin transfer buffer, pH 8.3 [25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol], that had been prechilled to 4°C.

Immunodetection of the immobilized proteins was carried out according to the manufacturer's instructions, using the ECL Western blotting kit (Amersham). Following incubation with the primary (rabbit anti-carboxylesterase, raised to peptide YMYGFQYRPSFS) and secondary (horseradish peroxidase-labeled anti-rabbit) antibodies, the membrane was placed in an autoradiography cassette and exposed to autoradiography film (Hyperfilm-ECL; Amersham) for 20 min. The film was developed in a Kodak X-OMAT M20 processor.

RESULTS

Transient Expression of Altered and Normal Human Carboxylesterase

Expression vectors containing the unaltered form of a human liver carboxylesterase (pRC/CMV-hCaE), a mutated carboxylesterase gene (pRC/CMV-mCaE), and vectors without insert DNA (pRC/CMV) were transfected into 293T cells; mock transfections (buffer only) were done as well. Following 72 h of incubation, cells and media were collected for assay of carboxylesterase activity. Figure 2 shows a comparison of the transient expression of the unaltered carboxylesterase gene (normal) and the substituted (Leu to Arg) carboxylesterase gene. Carboxylesterase activity was measured in both cell extracts and culture media. Total enzymatic activity for both groups was essentially the same; however, the relative distribution of the enzymatic activity differed markedly between the two groups. Cultures of cells transfected with the normal gene had an intracellular to extracellular activity ratio

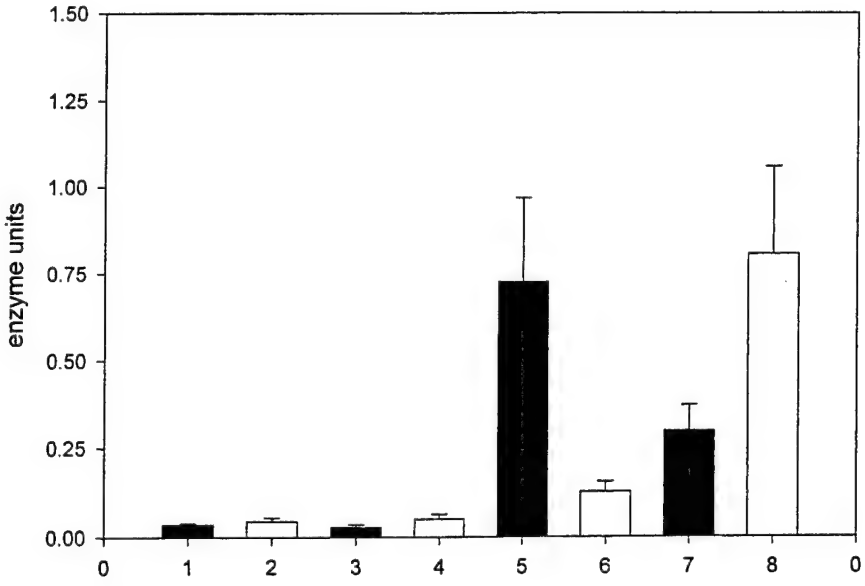

FIG. 2. Expression of carboxylesterase by transiently transfected 293T cells. 293T cells were transfected with buffer only (lanes 1 and 2), pRc/CMV (lanes 3 and 4), pRc/CMV-hCaE (lanes 5 and 6), or pRc/CMV-mhCaE (lanes 7 and 8). Shaded bars represent the intracellular enzyme levels/100-mm dish of each group, and clear bars represent the extracellular enzyme levels/100-mm dish of each group. Each bar shows the mean ± SE mean of seven experiments.

of approximately 9:1. In contrast, cells transfected with the mutated gene had an intracellular to extracellular ratio of 1:3. Thus, the conversion from leucine to arginine in the carboxy-terminal amino acid of carboxylesterase resulted in secretion of the enzyme into the media.

Expression of a Secreted Form of Human Carboxylesterase

After 48 h, 293T cells transfected with pRC/CMV-mCaE were grown in the presence of G418 to produce clones that continually express the altered carboxylesterase gene. A clone containing the normal gene was found to express a human carboxylesterase at levels similar to those observed for transient expression (see Fig. 2). Enzyme expression was measured in cultures of three clones bearing the mutated carboxylesterase gene. As depicted in Fig. 3, the carboxylesterase activities in the media were significantly greater than those of the transiently transfected cell cultures. The intracellular to extracellular activity ratios were similar to those seen in the transiently transfected culture. The intracellular levels of enzyme were the same for cultures bearing either carboxylesterase gene. However, the enzymatic activity was significantly higher in the media derived from the mutant clones.

Isoelectric Focusing Patterns

A comparison of the heterogeneity of the enzyme expressed by different 293T clones with that of human liver extract is given in Fig. 4. Samples from the media of cells containing the mutant gene are shown in lanes 2, 3, and 4. These show two major bands at p*I* 5.6–5.8, with minor bands at p*I* 5.5 and p*I* 6.1. In contrast, samples from the media of cells containing a normal gene, lane 5, and media from the mock transfected cells, lane 6, do not show detectable carboxylesterase activity. The forms of the enzyme present in extracts of cells are shown in lanes 7, 8, and 9. Similar banding patterns, as in the other samples, are seen with a major band at p*I* 5.6–5.7. Thus, the pattern of heterogeneity indicates similarity between the altered carboxylesterase gene, expressed and secreted from the cell, and the normal carboxylesterases expressed intracellularly in culture and in human liver extracts.

Hydrophobic Interaction and Dye-Ligand Affinity Chromatography

Based on the observation that carboxylesterase is a very hydrophobic protein, we decided to make use of the hydrophobic nature of this enzyme. Five different HIC media [phenyl-Sepharose high performance, phenyl-Sepharose 6 fast flow (low sub and high sub), butyl-Sepharose 4 fast flow, and octyl-Sepharose 4 fast flow] were tested, each with different hydrophobic characteristics. Chromatography on an octyl-Sepharose column, coupled with 50 mM octylglucopyranoside in the elution buffer, gave the best purification of carboxylesterase, compared to the other HIC media tested (data not shown). A three- to fivefold increase in specific

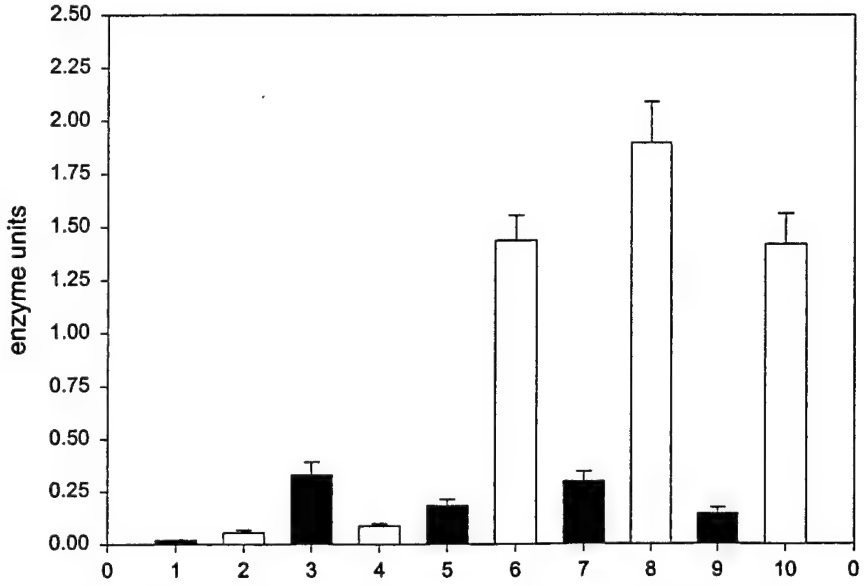

FIG. 3. Stable expression of carboxylesterase. Intracellular enzyme levels/100-mm dish (shaded bars) were compared to extracellular enzyme levels/100-mm dish (clear bars) for each of three mutant clones (clone A, lanes 5 and 6; clone B, lanes 7 and 8; clone C, lanes 9 and 10), as well as for 293T cells stably expressing normal human liver carboxylesterase cDNA (lanes 3 and 4) and 293T cells that were not transfected (lanes 1 and 2). Each bar shows the mean ± SE mean of nine experiments.

activity, along with an increase in total activity, was observed. However, HIC gave only partial purification. Albumin was the major contaminant (greater than 50% of the total protein) in the carboxylesterase sample.

To remove albumin following HIC, the enzyme sample was added directly onto a Cibacron blue column. The major contaminant, albumin, was reduced to less than 5% of the remaining total protein (Fig. 5). However, the carboxylesterase sample was still not pure.

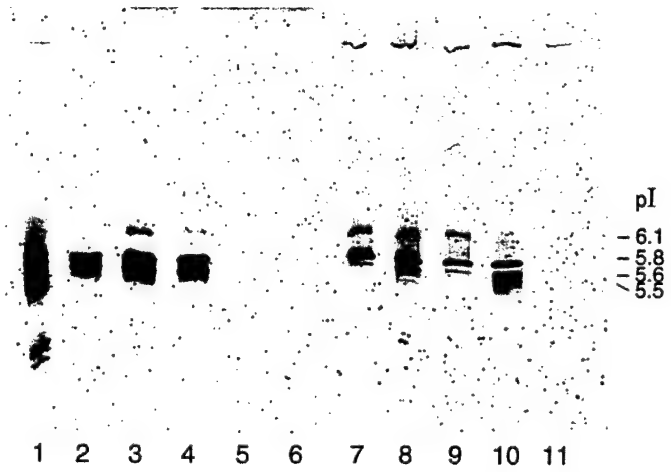

FIG. 4. Isoelectric focusing of carboxylesterase protein. Thirty micrograms of human liver protein (lane 1), 35 μg of total protein isolated from cell samples, and 200 μg of total protein isolated from media were loaded onto an Ampholine gel (pH 3.5–9.5). Media samples are shown in lanes 2–6 (clone C, lane 2; clone B, lane 3; clone A, lane 4; the normal clone, lane 5; nontransfected 293T, lane 6). Cell supernatants are shown in lanes 7–11 (clone C, lane 7; clone B, lane 8; clone A, lane 9; the normal clone, lane 10; nontransfected 293T, lane 11).

Western Blot

A nondenaturing polyacrylamide gel, containing the ammonium sulfate, HIC, and Cibacron blue fractions, was blotted onto a nitrocellulose membrane. To deter-

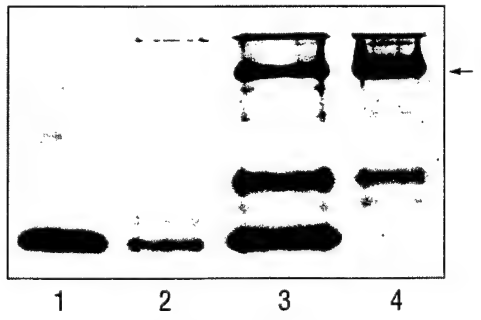

FIG. 5. Nondenaturing PAGE. Bovine albumin standard (5 μg) is shown in lane 1. The partial purification of carboxylesterase is shown in lanes 2–4 (ammonium sulfate precipitation, lane 2; hydrophobic interaction chromatography, lane 3; Cibacron blue affinity chromatography, lane 4). 2 μg of total protein for each sample was loaded onto the gel. The gel was stained for enzyme activity using α-naphthylacetate as substrate coupled to Fast Red TR salt. The enzyme activity band is denoted by the arrow. Following rinsing, the gel was stained for total protein with a 0.1% solution of Coomassie Blue R-250.

mine whether the inactive protein bands were denatured enzyme or contaminant proteins, immunodetection, using a polyclonal antibody to carboxylesterase, was performed. A single, dark, thick band of protein was detected in the HIC and Cibacron blue fractions (Fig. 6), which corresponds to the carboxylesterase activity band visualized on a nondenaturing polyacrylamide gel stained with α-naphthylacetate (Fig. 5). The ammonium sulfate fraction also shows a single band at the same position, but the band is very faint.

DISCUSSION

Although significant expression of an intracellular form of carboxylesterase was achieved in previous studies in our laboratory, the enzyme reached a plateau of expression. Increased expression of carboxylesterase, in culture, was desired to provide sufficient quantities for isolation and purification of the enzyme, which will be used in X-ray crystallography studies and in animal studies to evaluate the enzyme's potential as a prophylactic treatment of organophosphate toxicity. The present study shows the conversion of a membrane-bound form of a human liver carboxylesterase to a secretory enzyme by changing its C-terminal leucine to an arginine residue. In agreement with previous studies on KDEL-tagged proteins (30,39), substitution of the C-terminal leucine of a fully mature human liver carboxylesterase disrupts selective retrieval to the endoplasmic reticulum by the KDEL sorting receptor. Expression of the mutated carboxylesterase cDNA (pRc/CMV-mhCaE) was compared to expression of the normal cDNA (pRc/CMV-hCaE) stably integrated into the same cell line. After 3 days of incubation, intracellular levels of enzyme showed no differences between the extracts from cells stably transformed with either the normal or the mutated gene. However, the growth media of cells bearing the mutated gene had greater than five times the enzyme activity than that from cells bearing the normal gene. The transformed cells remained active in expressing the enzyme through 20 passages, and cells expressing the mutant carboxylesterase were equally viable compared to those expressing the normal enzyme as measured by trypan blue exclusion at the time of collection. Thus, the higher level of mutant carboxylesterase expression in the media is not the result of cell death.

Comparison of the mutant and normal carboxylesterase expression by 293T cells with carboxylesterase derived from human liver (see Fig. 4) showed similar patterns of heterogeneity, with no observable changes in activity, suggesting that the mutated carboxylesterase is very similar to the normal carboxylesterases expressed in culture and derived from human liver. Thus, the C-terminal mutation does not alter the functional characteristics of the enzyme.

The multiple banding pattern of carboxylesterase enzymes on isoelectric focusing gels has led to some controversy as to whether multiple forms of the enzyme or a single form of the enzyme exists in human liver. Southern blot analysis of rat and human genomic DNA (5) suggests the existence of multiple carboxylesterase genes in each species, although there are fewer carboxylesterase genes in humans. Individual carboxylesterase enzymes can produce microheterogeneity on isoelectric focusing gels. The observed microheterogeneity is self-generating (40) and may be the result of any of the following: ampholine–protein interactions (40,41), protein–protein aggregation (41), and/or variation in posttranslational modifications such as glycosylation (41,42).

The slight differences in banding pattern between the mutant and the normal carboxylesterase expressed in culture are most likely due to differences in the processing of the proteins through the Golgi (degree of glycosylation); the mutant protein being secreted and the normal protein remaining in the cell. Comparing the recombinant enzymes to the native enzymes in liver extract, the slight differences in heterogeneity can be explained by differences in posttranslational modification in the different cell types (kidney cells versus liver cells), as well as the existence of multiple carboxylesterase genes in the human liver. Liver extract on the zymogram exhibits the low-p*I* range enzyme as well as the mid-p*I* range enzyme, both exhibiting microheterogeneity and corresponding to the two distinct carboxylesterases purified from human liver by Ketterman *et al.* (40).

Once it was observed that the mutated cDNA facilitated increased expression of carboxylesterase in culture with no adverse effects on enzymatic activity, attempts to purify the enzyme were begun. Purification of carboxylesterase from cell culture proved to be a

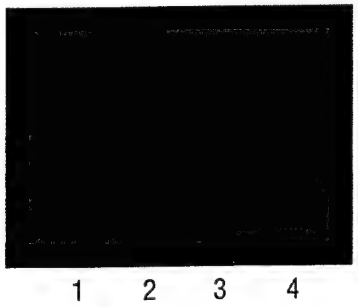

FIG. 6. Western blot. A nondenaturing polyacrylamide gel showing the partial purification of the mutated carboxylesterase was blotted onto a nitrocellulose membrane. The membrane was subsequently exposed to a polyclonal antibody to carboxylesterase and to a horseradish peroxidase-labeled secondary antibody. Chemiluminescence was carried out as described under Materials and Methods. Lane 1, albumin standard; lane 2, ammonium sulfate fraction; lane 3, HIC fraction; lane 4, Cibacron blue fraction.

TABLE 1

Purification of Carboxylesterase from the Culture Medium

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Overall yield (%)	Purification factor
$(NH_4)_2SO_4$	119	7.86	0.066	100	1
HIC[a]	12.28	4.64	0.378	59	5.72
Cib. blue	0.636	0.765	1.2	9.7	18.2
$(NH_4)_2SO_4$	156	12.89	0.083	100	1
HIC[b]	0.88	5.14	5.84	40	70.4
Cib. blue	0.145	1.1	7.58	11	91.3

[a] Sample eluted with 50 mM octylglucopyranoside in column buffer.
[b] Sample eluted with 1% Triton X-100 and 1% glycerol in column buffer.

very difficult task, in that the enzyme is very hydrophobic and will bind tightly to almost any support matrix. Several purification procedures were attempted with little success. However, two methods did give a high level of purification of the enzyme, while maintaining a high level of enzymatic activity.

Based on the observation that carboxylesterase is extremely hydrophobic, we decided to exploit the hydrophobic nature of this enzyme. Hydrophobic interaction chromatography on an octyl-Sepharose column gave the best purification of the enzyme compared to other techniques that were attempted. Increases in specific activity and total activity of the enzyme were observed. When the elution buffer for HIC was changed to include 1% glycerol and 1% Triton X-100, in place of octylglucopyranoside, a further increase in specific activity was observed (Table 1). HIC coupled with affinity chromatography on a Cibacron blue matrix increased the level of purification achieved by removing the major contaminant protein, albumin.

Previous studies (1,40) have reported on the purification of carboxylesterases from human liver tissue. The mutant carboxylesterase enzyme isolated from the culture media focused in the mid-p*I* range on a zymogram. Therefore, we believe that the enzyme we are expressing is similar to the mid-p*I* range liver enzyme isolated and purified by Ketterman *et al.* (40). Comparison of the recombinant protein purification with the purification of the native enzyme shows that similar levels of purity were achieved. The higher specific activity of the native enzyme (approximately 25 U/mg) is due to the larger amount of starting material: 8610–8994 mg of total protein from liver homogenate versus 100–200 mg of total protein from culture media. The level of purification achieved using octylglucopyranoside to disrupt hydrophobic interactions was similar to that achieved by Ketterman (18.2 versus 17.3, respectively). When Triton X-100 was used in place of octylglucopyranoside, higher levels of purification were achieved (Table 1): 91.3 up to 190 (data not shown). We have achieved a significant level of purification of the recombinant enzyme in just two steps. By exploiting the hydrophobic nature of the enzyme, and using nonionic detergents in the elution buffers, we were able to maintain a high level of total activity, which produced a high overall yield: 8–15% of the total starting activity in the final fraction.

A sample from each purification step was developed on a nondenaturing polyacrylamide gel. Each fraction showed the presence of contaminant proteins with a slightly higher molecular weight than albumin. It was thought that these protein bands could be denatured carboxylesterase enzyme, perhaps subunits, in different dissociation states, that were no longer active. Western blotting and immunodetection, using a polyclonal antibody to carboxylesterase, were done to determine if the inactive protein bands were, in fact, denatured enzyme. Immunodetection of the Cibacron blue fraction showed a thick, dark band of protein that corresponds to the major carboxylesterase activity band seen on a nondenaturing polyacrylamide gel. A thin band, above the major band, was also recognized by the carboxylesterase antibody. However, none of the bands below the major band were recognized by the antibody. Perhaps the denatured form of the enzyme was not recognized by the antibody. It is known that small changes in epitope structure can abolish antigen recognition (43). Structural differences between the active enzyme and the denatured enzyme may have led to differences in epitope structure, which would allow the antibody to recognize the active enzyme, but not recognize the denatured form of the enzyme.

Conversely, the protein bands below the major carboxylesterase band may not be denatured enzyme at all. They may be any number of proteins found in fetal bovine serum, which was used to supplement the cell culture medium. Stably transfected cells, secreting carboxylesterase, were grown in serum-reduced and serum-free culture medium, in an effort to minimize the number of contaminant proteins present. However,

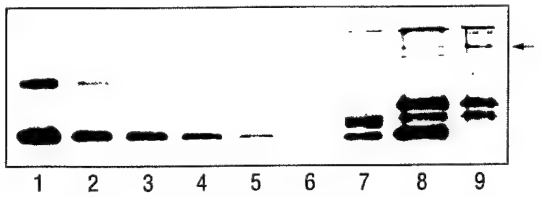

FIG. 7. Estimation of carboxylesterase purification. Carboxylesterase samples at different stages of purification were separated on a nondenaturing polyacrylamide gel and compared to different concentrations of an albumin standard. The gel was stained with a 0.1% solution of Coomassie Blue R-250. Lanes 1–6 contain the albumin standard (2 μg, lane 1; 1 μg, lane 2; 0.75 μg, lane 3; 0.5 μg, lane 4; 0.25 μg, lane 5; 0.1 μg, lane 6). The ammonium sulfate, HIC, and Cibacron blue fractions are shown in lanes 7, 8, and 9, respectively. 2 μg of total protein for each sample was loaded onto the gel. In the Cibacron blue fraction, the protein band corresponding to active carboxylesterase (designated by the arrow) was compared to the different concentrations of albumin to estimate the amount of protein that is active enzyme.

these cells did not grow very well, and the amount of carboxylesterase (total enzyme units) produced was significantly reduced. Thus, using serum-free or serum-reduced culture medium was not an option for increased expression of carboxylesterase.

By comparing the Cibacron blue fraction to different concentrations of an albumin standard on a nondenaturing polyacrylamide gel (Fig. 7), it was estimated that approximately 15% of the protein in the sample corresponded to active carboxylesterase enzyme. The sample contained 145 μg of total protein; thus, the amount of active enzyme was 21.75 μg. The sample had a total activity of 1.1 enzyme units and a specific activity of 7.58 units/mg. In order to produce 1 mg of active recombinant carboxylesterase enzyme, 50 175-cm^2 flasks of stably transformed 293T cells (each containing 30 ml of culture medium) would be required.

Stably transformed 293T cells will continuously express the carboxylesterase enzyme over several passages. This relatively constant expression of active enzyme indicates that stably transformed cells may be grown in a chemostat, which would allow for large-scale production of the mutant carboxylesterase. The production, isolation, and purification of sufficient quantities will facilitate the use carboxylesterase in X-ray crystallography studies and in animal studies to evaluate the enzyme's potential as a prophylactic treatment of organophosphate toxicity.

ACKNOWLEDGMENTS

This work was supported in part by U.S. Army MRICD DAAD 05-95-P-5139 and Biomedical Research Support Grant 5-07-RR5635-30.

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Expression and Purification of a Recombinant Secretory Form of Human Liver Carboxylesterase

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Abstract

Serine-dependent carboxylesterases (E.C.3.1.1.1) are found in a variety of tissues with high activity detected in the human liver. Carboxylesterases (hCaE) hydrolyze aliphatic and aromatic esters, and aromatic amides. Carboxylesterase may play an important role in the detoxification of xenobiotic chemicals that contain organophosphate (OP) compounds. Thus, an injectable form of human hCaE should prove to be a valuable antidote for protecting soldiers from these chemical agents. To this end, clones containing a site-mutated cDNA were prepared and used to stably transform human 293T cells. Transformed 293T cells were grown in a chemostat, and conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. In these studies, enzymatic activity was found to be optimal in cell culture medium at four hours. Enzymatically active carboxylesterase was isolated from the culture media, and the initial steps of enzyme purification were accomplished. Our results indicate that an active recombinant enzyme functions as native enzyme with respect to inhibition by organophosphates and reactivation by oximes.

This work was supported by the U.S. Army Medical Research and Material Command under Contract Order DAMD17-00-1-0518

Introduction:

The serine-dependent carboxylesterases (E.C. 3.1.1.1) are found in many different species (1-8), and in a variety of tissues (9-14), with high activities detected in the liver. Generally, carboxylesterases exist as 60 kDa monomers, but a few associate to form homotrimers of approximately 180 kDa (1-4). cDNA clones have been obtained by screening lambda gt11 expression libraries. Their screenings have resulted in the expression of five isoenzymes of rat liver carboxylesterase (5-9) and at least two isoenzymes from the human liver (10-12). The cDNA sequences are, generally, 1.7 to 1.9 kb in size, and they encode mature proteins that range from 507 to 568 amino acids. The different isoenzymes of liver carboxylesterases are all N-linked glycoproteins of the high mannose type (5,6,12,13). Core glycosylation of the carboxylesterases occurs in the endoplasmic reticulum lumen (14,15), and, thus it is necessary to stabilize the active conformation of the protein (6,16). Whereas, most glycosylated proteins are secretory, the carboxylesterases are localized to the luminal side of the endoplasmic reticulum, especially in humans (4-6). Very small amounts of liver carboxylesterase, however, are present in the serum (17). Carboxylesterases can hydrolyze a variety of substrates in the serum, including aliphatic and aromatic esters, and aromatic amides (18). An important function of these enzymes may be the hydrolysis, and subsequent detoxification, of pesticides, insecticides and drugs (18), many of which contain organophosphate compounds that bind covalently to the active site of the enzymes (19,20). In this regard, the carboxylesterases generally function as a high affinity-low capacity detoxification mechanism, in which organophosphates react in an irreversible 1:1 stoichiometry (17). Thus, the detoxifying ability of carboxylesterase is limited by its low concentration in serum where it encounters organophosphate compounds. A treatment for organophosphate toxicity is the administration of oximes, which reactivate inhibited acetylcholinesterase and restore cholinergic neurotransmission (17). Species with high levels of serum carboxylesterase, such as rats and mice, achieve a higher level of oxime-induced reactivation of organophosphate-inhibited acetylcholinesterase than species with lower levels of carboxylesterase; this suggests that oximes also reactivate organophosphate-inhibited carboxylesterase (17). This recycling of organophosphate-inhibited carboxylesterase provides additional protection by making the enzyme available for further binding to organophosphates, and thus, increased detoxification.

Methods

Construction of Expression vectors:

Carboxylesterase cDNA (GenBank Accession No. M55509) was subcloned from a pUC9 vector (10) into the pNotA plasmid (5'-3', Inc. Bolder, CO). Site directed mutagenesis was performed at the carboxy-terminus; the codon CTG for leucine was converted to CGC for arginine. The nucleotide change created a novel Eco47III restriction site. The carboxylesterase cDNA fragment was inserted into the expression vector The 1.9 kb Xba I fragement, containing the full-length mutated human carboxylesterase cDNA was ligated into the eucaryotic expression plasmid, pRc/CMV at the single XbaI site located at position 985. The presence of the carboxy-terminal mutation is shown at position 2748 (21).

Expression of carboxylesterase

Human embryonic kidney cells (293T) were stably transformed with the expression plasmid pRc/CMV-mhCaE plasmid, containing a secretory form of the human liver carboxylesterase and were grown in Dulbecco's Modified Eagles's Medium.(19). The culture media were supplemented with 10% fetal bovine serum. 2 mM glutamine, 10mM Hepes burffer, pH 7.5, and 50 I.U. penicillin-streptomycin/ ml. Cells were incubated at 37C in the presence of 5% CO_2. Aliquots of the culture media were periodically removed and assayed for the maximum appearance of carboxylesterase in the culture media. Fractions were assayed spectrophotometrically for enzyme activity with p-nitrophenylbutyrate as substrate (22) and analyzed for the presence of contaminating proteins using 12% non-denaturing polyacrylamide gel electrophoresis (23). Following electrophoresis, the gels were soaked in 0.1M sodium phosphate buffer; pH 7.4 for one hour, and the carboxylesterase activity was measured using alpha naphthylacetate as

substrate coupled to Fast Red TR salt (24). The gels were then rinsed with deionized water and stained for total protein with Coomassie blue R-250.Culture conditions for optimal secretion were determined to be 4 hours following introduction of cells to fresh culture media. Media was separated from cell cultures and pooled.

Hydrophobic Interaction Chromatography (HIC)

For the initial steps of enzyme purification, carboxylesterase was precipitated from the media using 70% saturated ammonium sulfate and separated from contaminating proteins by hydrophobic interaction chromatography (HIC) using octyl sepharose 4 fast flow (Sigma) as matrix material. The enzyme was eluted from the HIC media using 50 mM octylglucopyranoside. Fractions were again assayed for carboxylesterase activity and analyzed by nondenaturing gel electrophoresis.

Immunoprecipitation with anti-bovine serum albumin IgG

Following HIC chromatography, the sample was lyophilized and stored at –80C. 40-80 mg of lyophilized material was dissolved in 2.5 ml of phosphate buffer. The sample was the equilibrated in phosphate buffered saline (PBS) using a PD-10 column (Pharmacia). 3.5 ml of the supernatant was incubated with agarose-conjugated anti-BSA IgG for 30 minutes at 25 C. The mixture was centrifuged at 1200 rpm and washed a second time to remove remaining trapped enzyme from the antibody complex. The enzyme activity of the supernatant was measured and was analyzed by nondenaturing PAGE as previously described.

Preparative Isoelectric Focusing

Liquid preparative isoelectric focusing was performed using an LKB 8100 Ampholine Electrofocusing column as described by the manufacturer's manual. Approximately 25 mg of protein from the HIC chromatography was loaded onto a column containing Ampholine carrier ampholyted pH 5.0-8.0. For the intitial separation, the anode was at the bottom of the column. This resulted in the major protein band, bovine serum albumin, precipitating at the bottom of the column. The column was fractionated, and the carboxylesterase activity was measured (21). The peak fractions located around pH 5.3 were collected. The carboxylesterase peak was then loaded onto a second column where the anode and cathode were reversed; the cathode was at the bottom of the column. Samples were again fractionated and assayed for carboxylesterase activity.

Carboxylesterase Inhibition and Reactivation

Inhibition of hCaE was performed in 0.05 M phosphate buffer (pH 7.4) at 25^{0}C. Approximately 3 nmole of hCaE was incubated in the presence of either 1μM soman (methyl pinacolylphosphonofluoridate) or 10 μM physostigmine. After 30 minutes, the hCaE activity in the reaction mixture was assayed to confirm that >95% inhibition of hCaE had occurred. The inhibited hCaE was then separated from excess inhibitor by passing the reaction mixture through a TSK-GEL 20000SW column (300 mm x 7.5 mm; Thomson Instruments Co., Springfield, VA) equilibrated with phosphate buffer. The column was developed at a flow rate of 1.3 ml/min, and the inhibited hCaE eluted between 9 and 10 ml. The activity of each inhibited hCaE sample was compared to a corresponding control hCaE sample that was treated identically except for the exposure to the inhibitor. After chromatographic separation, a sample of inhibited hCaE was reactivated by addition of 1 mM diacetylmonoxime for 2 hours at 25^{0}C. Samples of the oxime reaction mixture were assayed for hCaE activity.

Results and Discussion

Stable clones of 293T cells, stably transfected with the plasmid pRC/mhCaE ( ), were placed in liquid culture for expression of the secretory form of human liver carboxylesterase. Carboxylesterase activity was observed to increase as early as 24 hours and to reach a maximum at day four (21). Cultured cells were separated from the media by low speed centrifugation and the pooled media from several cultures was used for the isolation of purification enzymatically active enzyme.

Carboxyleasterase was precipitated from the pooled media and precipitated using 70% saturated ammonium sulfate. The precipitate from the ammonium sulfate fraction was dissolved in 20 mM Hepes, pH 7.0 and applied to an octyl Sepharose (Pharmacia) column (21) for Hydophobic Interaction Chromatography (HIC). Previous observations had shown that HIC chromatography using octyl Sepharose, coupled with 50 mM octyglucophranoside in the elution buffer resulted in an enhanced partial purification of carboyxlesterase. However, the major contaminant of the preparation, bovine serum abumin, as well as several minor contaminants, were not removed by this procedure. *Figure 2, lane 4*, shows the presence of enzyme along with the bovine serum albumin and other minor contaminants from the HIC column. Anti-bovine serum albumin IgG was used to remove the bovine BSA during the purification procedure. The results of these experiments are seen in *Figure 2*. Lane 5 shows partial removal of bovine BSA; lane 6 is a second wash of the preparation to remove any remaining carboxylesterase from the antibody. Lanes 8 and 9 show a second treatment of the enzyme sample with anti bovine serum IgG. These results show that anti-BSA will remove a majority of the contamination BSA; however, there is a considerable loss of enzyme, which probably is a result of the binding to the matrix to which the anti-BSA is attached.

Based on our previous observations (21) that carboxylesterase binds to many matrices used in standard protein purification procedures, we developed a liquid isoelectric focusing preparative system. The strategy was to first remove the bulk of the BSA (pI 4.9) from the carboxylesterase (pI 5.3) by precipitation of the BSA into the anode solution at the bottom of the column (*Figure 3a*). The peak fractions containing carboxylesterase activity around pI 5.3 were collected and applied to a second column where the anode and cathode solutions were reversed. This resulted in the focusing of the BSA contaminant above the carboxylesterase fraction (*Figure 3b*). Non-denaturing page gel analysis of these peaks fractions from *Figure 3a and b* are shown in *Figure 3c and d*, respectively. The zymograms, lanes 1-3, show carboxylesterase activity while the Coomassie blue staining shows the relative amounts of enzyme and contaminating BSA protein. *Figure 3c* shows the presence of a small amount of BSA in the first isoelectric focusing fractionation while *Figure 3d*, following the second focusing column, shows almost complete removal of contaminating media proteins from the preparation.

Table 1 summarizes the effects of physostigmine and soman on the recombinant human liver carboyxlesterase produced from cDNA in human 293T cells. The data show a lack of inhibition by the general esterase inhibitor, physostigmine. The enzyme was about almost completely inhibited the carboxylesterase inhibitor, soman, and the inhibited recombinant enzyme was reactivated to greater than 90% activity by the oxime, diacetylmonoxime.

Conclusion:

Clones containing the secretory form of human liver carboxylesterase were used to establish culture conditions where the enzyme is produced maximally in the 293T human cell line. This objective was accomplished by growing cells in a chemostat where the cells are maintained in a constant exponential phase of growth. In addition, conditions were defined which allow for an optimal time of removal of the culture media for isolation of carboxylesterase. Enzyme was isolated from the culture media, and the initial steps of enzyme purification using ammonium sulfate precipitation, HIC chromatography, anti-bovine BSA IgG chromatography, and preparative liquid isoelectric focusing were accomplished. It was observed that

anti-bovine BSA IgG chromatography removed greater than 90 % of the contaminating protein, but resulted in a loss of enzyme and was not effective in removing minor contaminants from the cell culture media. The use of preparative liquid electrofocusing columns, however, was found to be efficient and resulted in a preparation that was free of bovine serum albumin and other minor contaminants as measure by polyacrylamide gel electrophoresis.

A major goal of this study was to produce a recombinant carboxylesterase that has the potential for use as an antidote against the action of soman and other toxic organophosphorus agents. For this purpose it was important that the recombinant enzyme, unlike the cholinesterases, possess a relatively specific esterase activity and that the recombinant enzyme be regenerable by oxime agents. In this regard, carboxylesterase can be differentiated from cholinesterase [20] by the inability of carbamates to inhibit carboxylesterase at concentrations that readily inhibit cholinesterase [25]. Additionally, carboxylesterase can be differentiated by its ability to be reactivated by oximes after inhibition by organophosphorus compounds; cholinesterase cannot be reactivated after inhibition by organophosphorus compounds. Physostigmine is the carbamate most commonly used to differentiate carboxylesterase from cholinesterase [22] and soman is the organophosphorus compound that has the greatest difference in oxime reactivation to differentiate inhibition of carboxylesterase and cholinesterase [25]. Thus, our findings show that the recombinant cDNA of human liver carboxylesterase codes for an enzyme that is processed in the Golgi to produce an active form of the enzyme in human cells. Most importantly, the recombinant carboxylesterase is not inhibited by physostigmine and it is almost completely regenerable by an oxime.

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Figures and Tables :

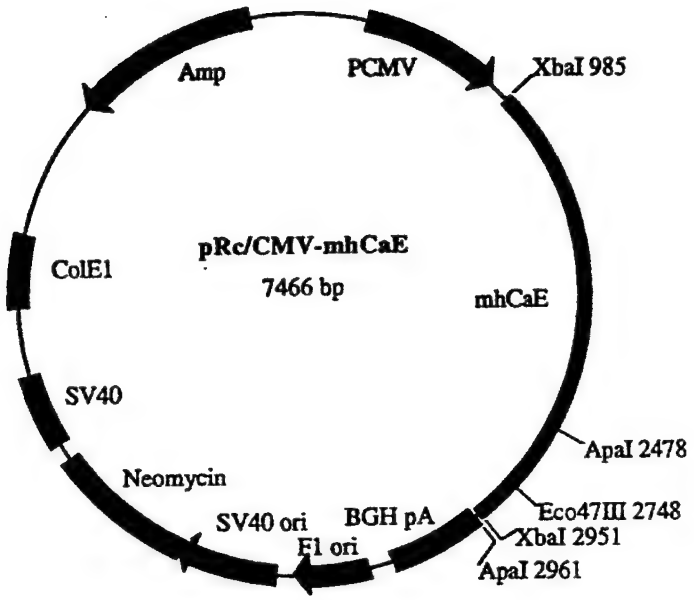


Figure 1: Expression vector containing the cDNA for secretory carboxylesterase

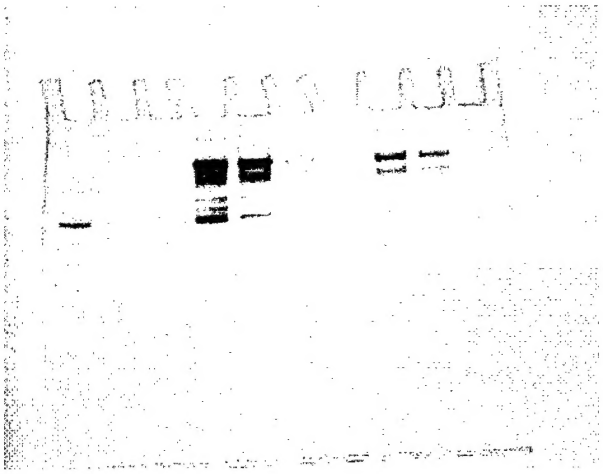


Figure 2. Nondenaturing PAGE. The lanes from left to right are lane 1; BSA standard, lane 2; urease standard, lane 3; blank, lane 4; the ammonium sulfate cut; lane 5-6; the anti-BSA IgG cut; lane 7; blank, lane 8 and 9; before and after a second treatment with anti-BSA IgG. Each lane shows a large molecular weight contaminant at the top of the gel followed by two major bands with significant carboxylesterase activity. The bottom band on the gel is BSA along with two minor bands located just above the BSA band. The gel was stained for enzyme activity using alpha naphthylacetate as substrate coupled to Fast Red TR salt. Following rinsing, the gel was stained for total protein with a 0.1% solution of Coomassie Blue R-250.

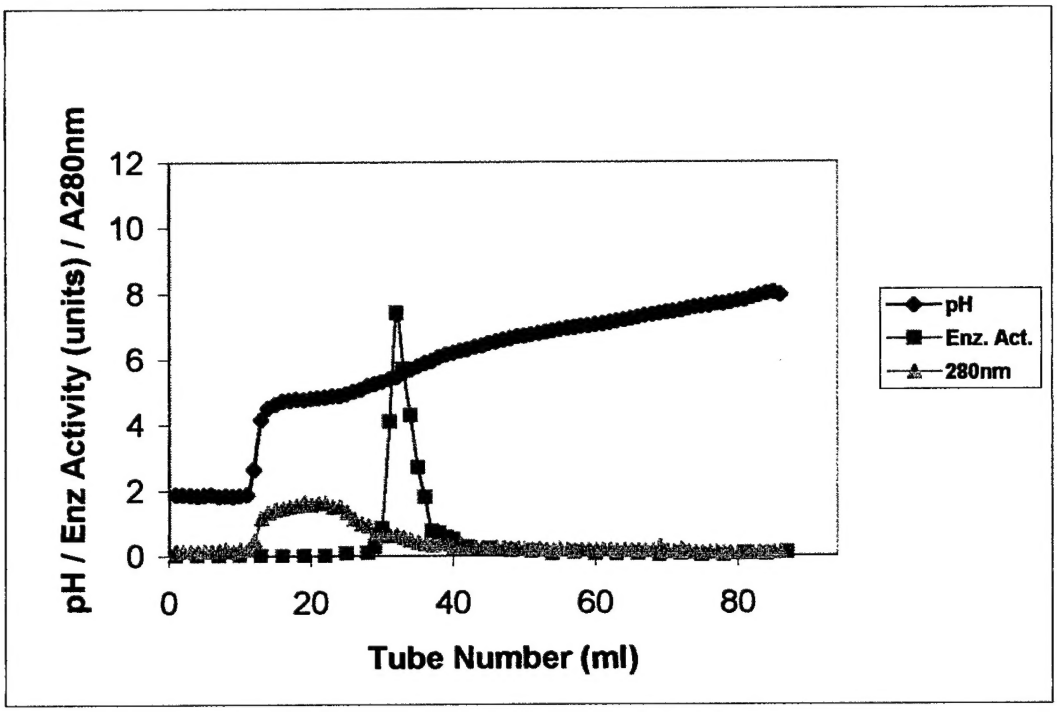


Figure 3a: Preparative Electrofocusing Chromatogram

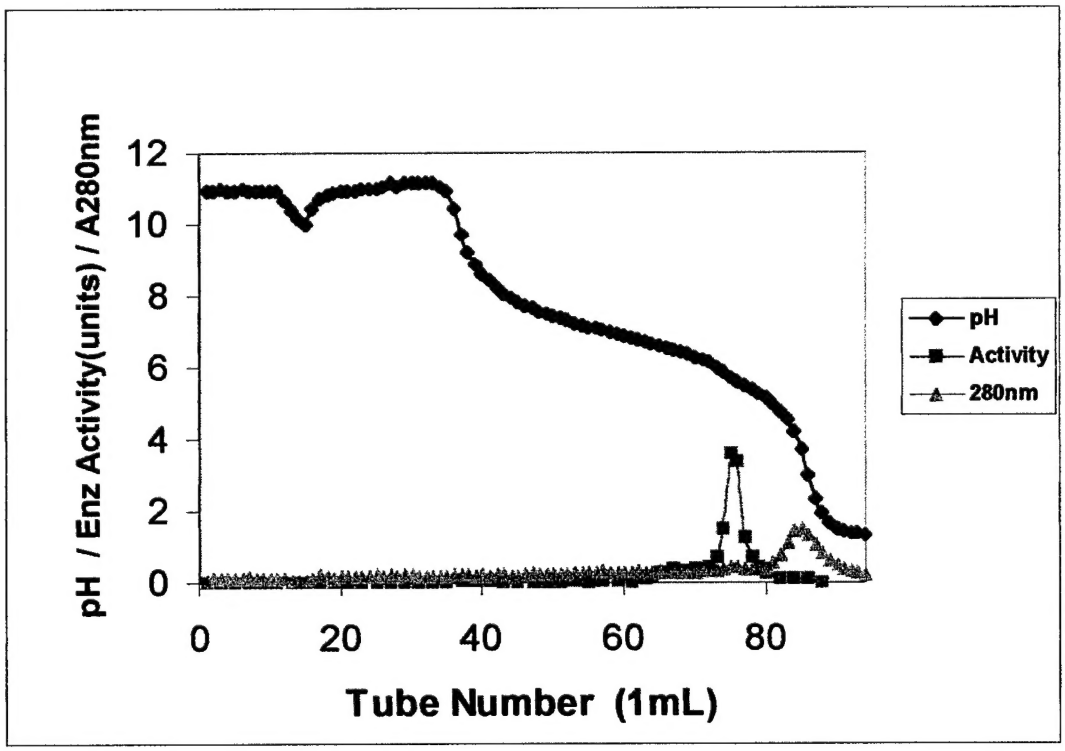
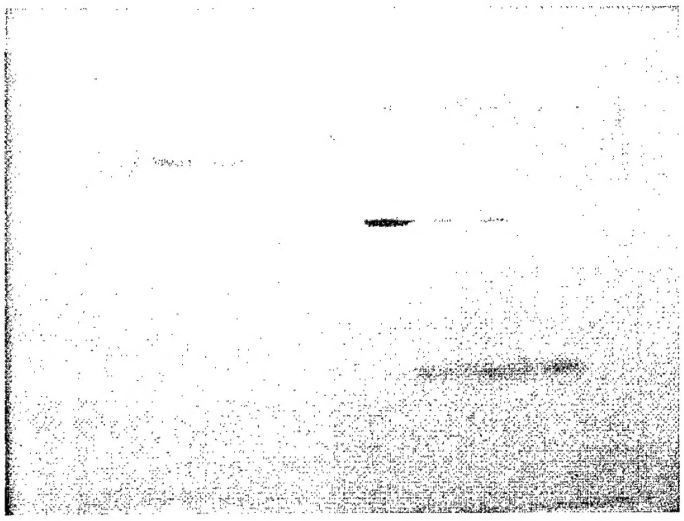
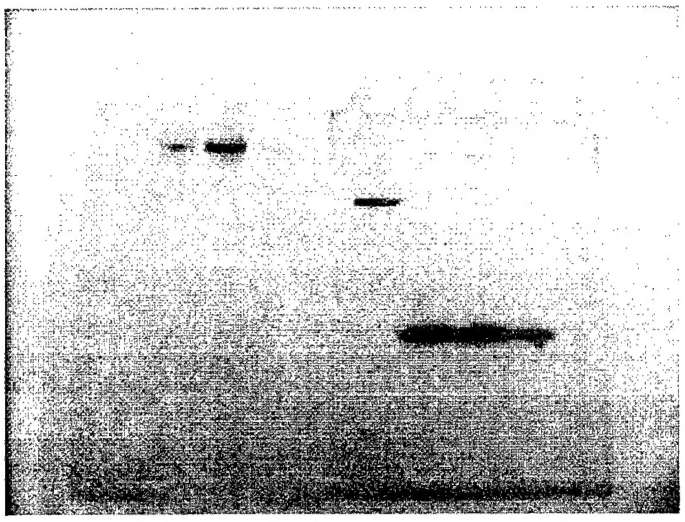


Figure 3b: Preparative Electrofocusing Chromatogram



1 2 3 4 5 6 7

Figure 3c: Nondenaturing Page Gel electrophoresis of fractions from figure 3a. Lanes 1-3; Zymogram showing enzyme activity from peak fractions 31,32,33. Lane 4; albumin standard, Lanes 5-6; Coomassie blue staining of peak fractions 31,32,33.



1 2 3 4 5 6 7

Figure 3d: Nondenaturing Page Gel electrophoresis of fractions from figure 3b. Lanes 1-3; Zymogram showing enzyme activity from peak fractions 74,75,76. Lane 4; albumin standard, Lanes 5-6; Coomassie blue staining of peak fractions 74,75,76.

Table 1: Inhibition and reactivation of recombinant Carboxylesterase

	Carboxylesterase Activity (% of Control)[a]
Treatment	**293T cells**
Physostigmine[b]	96+/-2
Soman[c]	8+/-4
Oxime[d]	91+/-4

[a] Activities (means +/- std. err.) are expressed relative to untreated extracts.

[b] Incubation with 10μM physostigmine for 30 minutes at 25°C (pH 7.4).

[c] Incubation with 1μM soman for 30 minutes at 25°C (pH 7.4).

[d] Incubation of soman-inhibited carboxylesterase with 1 mM diacetylmonoxime for two hours at 25°C (pH 7.4).